BRIEF COMMUNICATION

Multigenerational inbreeding in *Succisa pratensis*: effects on fitness components

F.X. PICÓ*[1], C. MIX**, N.J. OUBORG** and J.M. VAN GROENENDAEL**

*Center for Ecological Research and Forestry Applications (CREAF), Autonomous University of Barcelona, E-08193 Bellaterra, Spain**
*Department of Ecology, Radboud Nijmegen University, Toernooiveld 1, NL-6525 ED Nijmegen, The Netherlands***

Abstract

We examined the effects of repeated inbreeding on fitness components of the long-lived perennial *Succisa pratensis* (*Dipsacaceae*). Plants from six populations differing in size were used to establish lines with expected inbreeding coefficients f of 0, 0.5 and 0.75. The effects of different inbreeding levels were measured for seed set, seed mass, percentage germination and seedling relative growth rate. Seed set decreased following one generation of inbreeding and seedling growth rate decreased after two generations of inbreeding. Our study indicated that the mutational load is difficult to purge and that continued inbreeding tends to affect important traits in *S. pratensis*. Although the partial dominance hypothesis for inbreeding depression seems to account for the results, the overdominance hypothesis cannot be ruled out completely. Overall, we conclude that the response of a long-lived plant, such as *S. pratensis*, to repeated inbreeding does not differ from that of other plant species with shorter life spans, surely because the mechanisms that account for inbreeding depression are universal for all plant species.

Additional key words: deleterious mutations, inbreeding depression, purging, repeated inbreeding.

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Successive generations of inbreeding decrease hetero-zygosity, change genotype frequencies, reduce the amount of genetic variation within populations, and may result in inbreeding depression (McCall *et al*. 1994). Inbreeding depression, *i.e.* the reduction in fitness of selfed compared to outbred progeny, may strongly affect many life-cycle traits of plants (Husband and Schemske 1996). For this reason, inbreeding depression is considered as an important evolutionary force (Holsinger 1991, Lande *et al*. 1994, Charlesworth and Charlesworth 1999).

Studies of inbreeding depression in plants customarily compared the fitness of outbred progeny to that of progeny derived from only one generation of inbreeding. Nevertheless, there are multigenerational inbreeding studies that have been conducted on annuals, *e.g. Eichhornia paniculata* (*Pontederiaceae*) (Barrett and Charlesworth 1991), short-lived perennials, *e.g. Mimulus*

guttatus (*Scrophulariaceae*) (Dudash *et al*. 1997), *Impatiens capensis* (*Balsaminaceae*) (McCall *et al*. 1994) and *Plantago coronopus* (*Plantaginaceae*) (Koelewijn 1998), and to a lesser extent long-lived perennials, *e.g. Campanula rapunculoides* (Vogler *et al*. 1999). Hence, our knowledge on the effects of repeated inbreeding on plant fitness is still limited.

Studying the effect of different levels of inbreeding on fitness components can elucidate the genetic basis of inbreeding depression. It is accepted that inbreeding depression is largely caused by homozygosity of deleterious recessive alleles, *i.e.* the partial dominance hypothesis (Charlesworth and Charlesworth 1999, Carr and Dudash 2003), which allows plants to purge deleterious mutations from the population (Byers and Waller 1999). There is an alternative hypothesis where heterozygotes exhibit higher fitness than homozygotes, *e.g.*, the overdominance hypothesis, which does not result

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in effective purging of the inbreeding load (Charlesworth and Charlesworth 1999, Carr and Dudash 2003). Repeated inbreeding can reveal the effect of mutation on fitness traits as well as the way these mutations interact at different loci, *i.e.* epistasis, which may play a crucial role in evolution (Koelewijn 1998).

In this study, we examine the effects of different levels of inbreeding on fitness components of the long-lived, perennial herb *Succisa pratensis*. Results contribute to bridge the gap of knowledge we still have on the effects of repeated inbreeding on plant fitness, especially in the case of long-lived plants since experiments are time-consuming. Based on data obtained from a hand-pollination experiment, we determined the effects of three expected inbreeding coefficients ($f = 0$, $f = 0.5$ and $f = 0.75$) on fitness components including seed set, seed mass, percentage germination and seedling relative growth rate.

Succisa pratensis Moench. (*Dipsacaceae*) is a long-lived, perennial herb widely distributed in Europe. Detailed information on plant morphology, phenology and ecology can be found elsewhere (Mix *et al.* 2006). We selected six populations located across The Netherlands (around 52°1'N; 6°32'E) differing in population size. The population codes are SN (No. plants = 28 000), SH (25 000), SKD (15 000), SKM (1 500), SL (580) and SKL (50).

In late summer 1998, seeds from 15 randomly chosen plants were collected in each population. Seed storage and seed germination conditions followed Mix *et al.* (2006). One month after germination, one seedling per maternal plant (family hereafter) was individually potted (14 × 20 cm) and placed in a greenhouse (90 plants overall). In summer 2000, plants were self-pollinated. Part of the resulting inbred seeds was immediately sown to raise F_1 plants with one generation of inbreeding. The number of families per population that produced F_1 progeny were 9, 8, 8, 6, 6 and 6 for the SN, SKL, SH, SKM, SKD and SL populations, respectively. In summer 2001, F_1 plants and their respective mother plants were hand-pollinated. The soil of the flowerpots was renewed to avoid nutrient deficiency. We produced outbred (F_0, expected inbreeding coefficient $f = 0$) and inbred (F1, $f = 0.5$) progeny from the mother plants, and F_1 plants were self-pollinated to obtain seeds with two generations of inbreeding (F_2, $f = 0.75$).

To produce inbred and outbred progeny, four flower heads of each mother plant were selected (total 258 flower heads). Two of them were self-pollinated with pollen of the same plant while the other two were cross-pollinated with pollen of different plants within the same population. On F_1 plants, two flower heads were also self-pollinated. All hand-pollination procedures followed Mix *et al.* (2006).

In September 2001, seeds from all three hand-pollination treatments (around 3500 seeds) were collected for families that produced matched progeny (outbred seeds, seeds with one generation of inbreeding, and seeds with two generations of inbreeding). The final number of families with matched progeny was 8, 6, 5, 4, 3 and 3 for the SKL, SN, SKM, SKD, SH and SL populations, respectively. All resulting seeds were counted, and filled seeds were counted, separated and weighed. The seed set (filled seeds/total seeds) and individual seed mass (total mass of filled seeds/number of filled seeds) per flower head were calculated. Filled seeds (10 - 61 seeds per family and treatment) were potted (15 × 15 cm) and percentage germination recorded one month after sowing. In winter 2002, five seedlings per family and treatment (overall 60 seedlings, see below) were individually potted (15 × 15 cm). Immediately after transplantation, the number of leaves and the length of the largest leaf were recorded. The same measures were recorded three weeks after transplanting. The seedling relative growth rate (RGR) was computed as the difference between products of the log number of leaves and the log length of the largest leaf between censuses (as in Mix *et al.* 2006).

We only used one of the two flower heads per treatment and family in the analyses because some hand-pollinations failed in some families and treatments (45 of 258 pollinations failed). When both flower heads per treatment succeeded, we selected the flower head with the highest seed set because this indicated the maximum seed set value that plants could achieve under each treatment. We discarded those families on which the two hand-pollinated flower heads per treatment failed. As a result, the final number of families included in the analyses was 29 (3 - 8 families per population across the six populations of study).

The effect of inbreeding level and population (fixed factors) on seed set was analyzed with a two-way *ANOVA*. To examine the effect of inbreeding level and population on seed mass and percentage germination, we performed a two-way *ANCOVA* with seed set as the covariate. When appropriate, differences between inbreeding levels or populations were tested with Fisher PLSD post-hoc tests. Given the low germination rates obtained (most families produced 1 - 3 seedlings in one or two treatments) we could not use the same design to test the effects of inbreeding level and population on seedling establishment. In fact, only four of 29 families used in the experiment produced more than five seedlings per treatment. Consequently, one-way *ANOVA*s testing for the effect of inbreeding level on seedling RGR were performed separately for each family. When appropriate, variables were arcsin-square root transformed (for seed set and percentage germination) or log-transformed (for seed mass and seedling RGR) prior to analysis to meet *ANOVA* assumptions.

The relationship between fitness components and inbreeding level was analyzed by calculating the linear and quadratic terms of the relationship between log-transformed fitness components and inbreeding level (Koelewijn 1998). A significant nonlinear term of the quadratic regression indicates that the effects of mutations do not act in a multiplicative manner. Nonlinear negative coefficients mean that reinforcing epistasis is occurring whereas nonlinear positive

Table 1. Two-way *ANOVA* and *ANCOVA* for inbreeding level and population on seed set, seed mass and percentage germination of *Succisa pratensis*. Seed set is used as a covariate. Degrees of freedom (*df*) and *F*-values are given. Significance: *** - $P < 0.001$; ** - $P < 0.01$; * - $P < 0.05$; ns - non-significant.

Source	df	Seed set F	df	Seed mass F	df	Germination F
Seed set	−	−	1	18.08***	1	7.83**
Inbreeding level (I)	2	3.76*	2	0.42ns	2	1.09ns
Population (P)	5	1.21ns	5	2.94*	5	1.59ns
I × P	10	1.43ns	10	0.63ns	10	0.60ns
Error	81		65		65	

coefficients can be due to diminishing epistasis and/or purging of partially deleterious alleles (Koelewijn 1998). We performed quadratic regressions between inbreeding level and those traits that showed a significant inbreeding level effect.

Seed set significantly differed between inbreeding levels (Table 1). Outcrossing ($f = 0$) led to seed set values significantly higher than those of the two levels of inbreeding (Table 2*A*). Seed mass and percentage germination were not significantly affected by inbreeding levels (Table 1). On average, seeds weighed around 2 mg and percentage germination was around 22 % (Table 2*A*). There was no significant interaction between inbreeding level and population, and only seed mass significantly varied between populations (Table 1). In fact, population SH and SKL exhibited the highest seed mass values (mean ± SE = 2.66 ± 0.15 and 2.06 ± 0.07 mg for population SH and SKL, respectively), which significantly differed ($P < 0.05$) from the rest of populations (mean seed mass ranged 1.85 - 1.99 mg). Seed set, the covariate in the *ANCOVA*s, significantly

affected seed mass and percentage germination (Table 1), and the relationship between the covariate and both traits was negative (regression coefficient $r = -0.54$ and -0.32 for seed mass and percentage germination, respectively).

The examination of the effects of inbreeding levels on seedling RGR using one-way *ANOVA*s separately for each of the four families showed that two families from different populations significantly differed between inbreeding levels (SH - 24, $P < 0.05$; SKL - 15, $P < 0.01$), whereas the other two did not (SH - 6 and SKL - 21, $P > 0.05$). When differences between inbreeding levels were significant, results showed that seedling RGR values of $f = 0.75$ progeny were lower than those of $f = 0$ and $f = 0.5$ progeny, which did not significantly differ from each other (Table 2*B*).

Linear and quadratic regression analyses using expected inbreeding coefficients to predict fitness components were performed for seed set by pooling all plants from all populations since the population effect was non-significant (Table 1). Results revealed significant negative linear ($r = -0.61$, $P < 0.05$) and positive quadratic ($r = 0.14$, $P < 0.05$) coefficients for seed set. The significant negative linear coefficient agrees with the ANOVA results above, as seed set decreased with inbreeding level. In contrast, the significant positive quadratic coefficient indicated the existence of diminishing epistasis.

The results showed that, for seed set, the progeny from the two levels of inbreeding performed consistently less well than the outbred progeny, and that there were no differences between the two generations of inbreeding. This result suggests that continued inbreeding up to two inbred generations is insufficient to further reduce the genetic load for seed set. Our results agree with accepted theory stressing that mildly deleterious mutations would be hard to purge in long-lived perennials (Byers and Waller 1999). This conclusion is also supported by the significant negative linear coefficient that indicated the decline in seed set with increasing inbreeding level. Although the positive quadratic coefficient was also significant, which would indicate effective purging of deleterious alleles as inbreeding level increases (Koelewijn 1998), the linear coefficient provided a significantly better fit indicating that purging is unlikely to occur.

Table 2. *A* - Seed set, seed mass, and seed germination for each inbreeding level. *B* - Seedling relative growth rate, RGR; [cm d^{-1}] for four individuals in each inbreeding level. Different letters indicate that means (± SE) significantly differed from one another (Fisher PLSD post-hoc test, $P < 0.05$).

A

Variable	Inbreeding level $f = 0$	$f = 0.5$	$f = 0.75$
Seed set [%]	54.40 ± 3.30a	41.20 ± 4.70b	43.70 ± 4.30b
Seed mass [mg]	1.97 ± 0.10	2.03 ± 0.09	2.09 ± 0.08
Germination [%]	22.10 ± 3.50	23.70 ± 4.20	19.10 ± 3.30

B

Plant	Inbreeding level $f = 0$	$f = 0.5$	$f = 0.75$
SH - 24	5.7 ± 0.3a	5.8 ± 0.6a	3.2 ± 0.9b
SH - 6	4.6 ± 1.2	2.6 ± 0.4	3.1 ± 0.4
SKL - 15	4.8 ± 0.6a	4.1 ± 0.3a	1.9 ± 0.3b
SKL - 21	2.8 ± 0.2	2.2 ± 0.3	3.1 ± 0.7

It is widely accepted that the partial dominance hypothesis represents the main genetic basis of inbreeding depression. Under this hypothesis, outbred progeny are expected to perform better than inbred progeny because recessive alleles are masked in a heterozygous condition (Charlesworth and Charlesworth 1999, Carr and Dudash 2003). The overdominance hypothesis represents the alternative hypothesis in which heterozygote advantage is broken down at many loci over generations of inbreeding (Charlesworth and Charlesworth 1999, Carr and Dudash 2003). In the case of *S. pratensis*, it has been shown that there was no purging for seed set from one to two generations of inbreeding. Hence, purging of deleterious recessive mutations alone (*i.e.*, the partial dominance hypothesis) cannot explain the patterns of inbreeding depression for seed set observed in this study (see also Latta and Ritland 1994). Other multigenerational inbreeding studies also indicated that results are congruent with the partial dominance hypothesis but overdominance cannot be ruled out completely (Dudash *et al.* 1997, Koelewijn 1998).

The growth rate of *S. pratensis* seedlings also showed significant differences between inbreeding levels. When significant, seedling growth rate decreased after two generations of inbreeding but not following one generation of inbreeding. These results suggest that inbreeding depression for seedling growth rate is determined by deleterious alleles that need more than one generation of inbreeding to affect this trait. These results are consistent with other multigenerational inbreeding studies in plants showing significant family-level variation in inbreeding depression (Dudash *et al.* 1997, Koelewijn 1998, Picó *et al.* 2004). Variation among maternal families in inbreeding depression can be expected if plants differ in the number of recessive alleles that they carry, which is determined by the differential accumulation of mutations among maternal families (Koelewijn 1998).

Seed mass and percentage germination were not significantly affected by inbreeding levels. Interestingly, there was a trade-off between seed set and these two traits: high seed sets led to low seed mass and low germination rates, surely as a result of an internal reallocation of resources. Hence, inbreeding depression decreased seed set in *S. pratensis* but such a decrease led to increasing mass and germination rates of inbred seeds. This trade-off could cancel out the negative effects of inbreeding depression on seed set in the short term. However, inbred plants may carry deleterious mutations that significantly decrease fitness by reducing seedling growth rates following two generations of inbreeding.

Overall, we conclude that the response of a long-lived plant, such as *S. pratensis*, to repeated inbreeding does not differ from that of other plant species with shorter life spans, surely because the mechanisms (*i.e.* the partial dominance and/or overdominance hypotheses) that account for inbreeding depression are universal. Nevertheless, the effects of repeated inbreeding on population performance will appear faster in short-lived than in long-lived plants as generation time is shorter for the former than for the latter.

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